Profound changes in dopaminergic neurotransmission in the prefrontal cortex following flattening of the diurnal glucocorticoid rhythm: implications for bipolar disorder

Glucocorticoid diurnal rhythm and prefrontocortical dopamine

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ABSTRACT

Patients with bipolar disorder have abnormalities in glucocorticoid secretion, dopaminergic neurotransmission, and prefrontal cortical function. We hypothesised that the flattening of the diurnal glucocorticoid rhythm, commonly seen in bipolar disorder, modulates dopaminergic neurotransmission in the prefrontal cortex leading to abnormalities in prefrontally-mediated neurocognitive functions. To address this hypothesis, we investigated the effects of a flattened glucocorticoid rhythm on i) the release of dopamine in the prefrontal cortex and ii) the transcription of genes in the ventral tegmental area (VTA) coding for proteins involved in presynaptic aspects of dopaminergic neurotransmission.

Male rats were treated for 13-15 days with corticosterone (50µg/ml in the drinking water) or vehicle (0.5 % ethanol). Corticosterone treatment resulted in marked adrenal atrophy and flattening of the glucocorticoid rhythm as measured by repeated blood sampling. Animals treated with corticosterone demonstrated markedly enhanced basal dopamine release in the prefrontal cortex as measured by microdialysis in the presence of a dopamine reuptake inhibitor. Depolarisation-evoked release was also enhanced, suggesting that the corticosterone effect on basal release did not result from an increase in the neuronal firing rate. Local blockade of terminal D₂ autoreceptors failed to normalise release to control values, suggesting that enhanced release was not due to reduced autoreceptor sensitivity. In situ hybridisation histochemistry revealed that mRNAs coding tyrosine hydroxylase and the vesicular monoamine transporter2 were elevated in the ventral tegmental area of corticosterone treated rats.

Our data reveal that flattening of the glucocorticoid rhythm increases dopamine release in the prefrontal cortex possibly as a result of increased synthesis and vesicular storage. This provides a mechanistic explanation for prefrontal dysfunction in bipolar and other affective disorders associated with glucocorticoid dysrhythmia.

Key words: dopamine, ventral tegmental area, microdialysis, in situ hybridisation histochemistry, HPA axis, corticosteroid, corticosterone
INTRODUCTION

There is now compelling evidence that dopaminergic dysfunction is an underlying pathophysiological feature of the affective disorders, and in particular bipolar disorder. One of the key strands of evidence is derived from pharmacological data, collected over the last quarter of a century, detailing the effects of various drugs on mood and cognitive functions in healthy subjects and bipolar patients. Thus, direct and indirect dopamine receptor agonists have been shown to precipitate manic episodes in bipolar patients (Brook and Cookson, 1978; Gerner et al, 1976; Kemperman and Zwanikken, 1987; Van Kammen and Murphy, 1975; Vlissides et al, 1978), whilst antipsychotic drugs which block D₂ receptors (Kapur and Mamo, 2003; Wilson et al, 1998) have efficacy in stabilising mood symptoms during manic and depressive bipolar episodes (Brambilla et al, 2003; Surja et al, 2006; Wijkstra et al, 2006). More recently, it has been shown that depletion of the dopamine precursor tyrosine, in bipolar subjects can reduce the symptoms of mania (McTavish et al, 2001) and, in healthy subjects, can induce disrupted affect/reward characteristics which parallel those seen in bipolar depression (McLean et al, 2004).

The prefrontal cortex (PFC) is associated with working memory and executive functions (Dalley et al, 2004) and, more recently, has been implicated in emotional regulation via sub-cortical afferents (Beauregard et al, 2001; Levesque et al, 2003; Morgan et al, 2003; Ochsner et al, 2004; Ohira et al, 2006; Urry et al, 2006). Deficits in PFC mediated working memory and executive functions, in addition to the cardinal symptoms of emotional dysregulation, are characteristic of patients with both bipolar and unipolar affective disorders (DeBattista, 2005; Kaiser et al, 2003; Kolur et al, 2006; Lampe et al, 2004; Malhi et al, 2007; Martinez-Aran et al, 2004; Porter et al, 2007; Quraishi and Frangou, 2002; Savitz et al, 2005; Thompson et al, 2005). Furthermore, imaging studies have revealed abnormalities in PFC biochemistry and function in patients with bipolar disorder (Blumberg et al, 2003; Robinson et al, 2008).

The PFC receives inputs from the ventral tegmental area (VTA) via the mesocortical dopaminergic projection system. Dopaminergic neurotransmission in the PFC has a complex modulatory influence on mnemonic and executive functions mediated by this region (Arnsten, 1997; Floresco and Magyar, 2006; Mizoguchi et al, 2004; Robbins, 2005), in addition to regulating emotional aspects of PFC function (Fernandez Espejo, 2003; Morrow et al, 1999). In the context of the PFC functional deficits characteristic
of bipolar patients, these data suggest a potential role for prefrontal dopaminergic dysfunction in the pathophysiology of bipolar disorder.

Clinical studies have demonstrated that a common feature of affective disorders is disruption to the diurnal pattern of hypothalamic-pituitary-adrenal (HPA) axis activation. This disruption, which takes the form of an elevated basal plasma cortisol level, and a flattening of the diurnal rhythm, is particularly evident in severe/psychotic unipolar depression and bipolar disorder (Akesode et al., 1976; Cervantes et al., 2001; Deuschle et al., 1997; Linkowski et al., 1994; Sachar, 1975; Yehuda et al., 1996). Glucocorticoids have been linked to dopaminergic abnormalities in severe affective disorders (Schatzberg et al., 1985) on the basis of evidence that dopamine neurotransmission can be regulated by glucocorticoids. For example, in rats, adrenalectomy decreases dopaminergic neurotransmission in terminal projection regions (Barrot et al., 2001; Barrot et al., 2000; Biron et al., 1992; Faunt and Crocker, 1988, 1989; Mizoguchi et al., 2004; Piazza et al., 1996; Shoaib and Shippenberg, 1996; Tanganelli et al., 1990), while there is some evidence that chronic high dose corticosterone increases dopaminergic function (Czyrak et al., 2003). While manipulations such as those used in the above studies establish the principle that dopaminergic function is glucocorticoid sensitive, they do not necessarily predict the consequences of the subtle glucocorticoid dysregulation seen in affective disorders. Hence, the hypothesis that glucocorticoid dysrhythmia is causative in the development of mesocortical dopamine dysfunction in mood disorders (and particularly bipolar disorder) remains untested.

Here we determined the effect of a subtle elevation and flattening of the diurnal glucocorticoid rhythm on various functional aspects of the rat mesocortical dopaminergic system. Using a treatment protocol validated by repeated blood sampling, rats were administered a modest dose of corticosterone in their drinking water, over a two-week period, to flatten the glucocorticoid rhythm. We employed in vivo microdialysis to measure basal and stimulated dopamine levels in the PFC, and in situ hybridisation histochemistry with autoradiography to determine the expression of mRNAs coding for a variety of proteins involved in the synthesis, release, reuptake and metabolism of dopamine.
METHODS

Drugs and chemicals

Corticosterone (Sigma, UK) was dissolved in ethanol (Fischer Scientific, UK). Bupropion (Sigma, UK) and sulpiride (Sigma, UK) were dissolved in aCSF. Urethane (Sigma, UK) was dissolved in water for injection. All other chemicals were of analytical or high laboratory grade.

Animals and corticosterone treatment

Male Lister hooded rats (Charles River, UK), housed in groups of four, were used throughout.

Validation of corticosterone treatment protocol

Two groups of animals were prepared for continuous blood sampling to monitor the effects of corticosterone and vehicle treatment regimes on the diurnal corticosterone rhythm. All animals underwent ‘sham surgery’ for a corticosterone treatment protocol involving implanted pellets (data not reported here). This involved a small skin incision and suturing under general anaesthesia with isoflurane. The procedure took only a few minutes and animals were returned to group housing afterwards. Beginning the day following surgery, animals were treated with corticosterone (50 µg/ml) in the drinking water, or vehicle (0.5% ethanol) in drinking water. Animals had ad lib access to the drinking water.

Blood sampling and hormone assays were conducted as described elsewhere (Windle et al, 1998). Briefly, on the tenth day of treatment animals were anaesthetised and the jugular vein was cannulated, with the cannula exteriorised and attached to a steel spring screwed to the skull. Following cannulation, animals were singly housed and 4 days later (day 14 of treatment) cannulae were connected to a blood sampling system. Samples of whole blood (20 µl diluted in 100 µl heparinised saline) were collected every 20 min over more than 24 hours. Samples were pooled for corticosterone analysis by radioimmunoassay.
Measures of dopaminergic function

All animals were treated for between 13 and 15 days with corticosterone or vehicle in their drinking water as described above. The volume of water consumed by each cage of animals was recorded and animals were weighed regularly to monitor their health. Note: these animals did not undergo sham surgery or surgery for cannulation.

In vivo microdialysis

Following corticosterone (n=12) or vehicle (n=12) treatment for 13–15 days, animals were anaesthetised with urethane (1500 mg/kg) and fixed in a stereotaxic frame (David Kopf, USA) in the flat skull position. A concentric microdialysis probe (4.5 mm dialysing window; AN69 Hospal membrane) was stereotaxically implanted in the PFC (3.0 mm rostral and 0.7 mm lateral of bregma and -5.5 mm below the dura surface). The probe was perfused with artificial cerebrospinal fluid (termed ‘standard aCSF’) (composition: 140 mM NaCl; 3 mM KCl; 1 mM MgCl₂; 1.2 mM NaHPO₄; 0.27 mM NaH₂PO₄; 7.2 mM glucose; 2.4 mM CaCl₂) containing bupropion (30 µM) at a flow rate of 1 ml/min. Dialysate samples were collected every 20 min and dopamine content was measured using HPLC with electrochemical detection (Coulchem II, ESA Analytical, UK; Cell 1 +100 mV; Cell 2 +350 mV). Mobile phase for HPLC comprised 83 mM NaH₂PO₄, 0.84 mM EDTA, 0.46 mM OSA, 15% methanol in deionised water, pH = 4.0.

Once a steady baseline of dopamine, of at least 60 min duration, was obtained with standard aCSF, depolarisation evoked release was induced by switching the perfusion for ‘high K⁺ aCSF’ which contain 100 mM K⁺, for a period of 20 minutes. Then, following a further 60-100 min perfusion of standard aCSF, the perfusion was switched to ‘sulpiride aCSF’ which contained the D₂/D₃ receptor antagonist sulpiride (10 µM) for 80 min, followed by ‘high K⁺/sulpiride aCSF’ which contained both 100 mM K⁺ and sulpiride (10 µM) for 20 min. Finally the perfusion was again switched to sulpiride aCSF. Note: all aCSF contained bupropion (30 µM).

At the end of the experiment, animals were sacrificed by anaesthetic overdose and one adrenal gland was removed and weighed.
Tissue collection and sectioning for in situ hybridisation histochemistry

Two groups of animals (n=8 per group) were treated for 14 days with corticosterone or vehicle as described previously. At the end of treatment, animals were sacrificed by overdose with sodium pentobarbital (0.7 ml/kg i.p., 20% w/v solution, Dolethal, Vetoquinol UK). Note: all animals used for in situ hybridisation histochemistry had received the DOPA decarboxylase inhibitor NSD1015 (100 mg/kg i.p.) 20 minutes prior to sacrifice to allow analysis of tyrosine hydroxylase activity (data not reported here). Following sacrifice, the brain was removed, divided coronally, and the portion containing the midbrain was snap frozen in isopentane on dry ice. In a subset of these animals (n=4 per group) one adrenal gland was removed, dissected from surrounding tissue, and weighed.

Brain tissue was stored at -80 °C prior to sectioning. Sections (12 µm thick) were cut on a cryostat and thaw mounted onto RNAase free gelatin-subbed slides. Adjacent sections were mounted across a series of slides; 4 sections per slide were collected. Mounted sections were stored frozen at -80 °C until pretreatment. Sections were fixed in 4% paraformaldehyde, acetylated in 0.25% acetic anhydride, defatted in chloroform and dehydrated in ethanol before being air-dried and stored at -20 °C.

In situ hybridisation histochemistry

Oligonucleotide probes were synthesised by MWG-Biotech AG (Germany). Sequences were as follows:

Tyrosine hydroxylase (TH): 36 base probe sequence complimentary to bases 1380 to 1415 of the rat TH gene (L22651 ((Anton et al., 1994)): 5’ GGG AGA ACT GGG CAA ATG TGC GGT CAG CCA ACA TGG 3’;

vesicular monoamine transporter 2 (VMAT2): 48 base probe sequence complimentary to bases 271 to 318 of the rat VMAT2 gene (NM_013031 ((Schwartz et al., 2003)): 5’ ATG CCT TTA GGT CTG GTG GTC TGG TCT CGA GCA CCA GAG GTG GAG GCT 3’;

dopamine transporter (DAT): 43 base probe sequence complimentary to bases 1015 to 1057 of the rat DAT gene (M80233 ((Kilty et al, 1991)): 5’ GAG AAG GCA ATC AGC ACT CCA AAC CCA ACG CCG AGG GAG AAG C 3’;

monoamine oxidase A (MAO-A): 36 base probe sequence complimentary to bases 1591 to 1626 of the MAO-A gene (XM_001058993): 5’ AAG ATA CGC AAA TTC CCG AGC AGT TTT TGT CCA ACA 3’;

D2 receptor: 36 base probe sequence complimentary to bases 787 to 820 of the rat D2 receptor gene (X17458 ((Monsma et al, 1989)): 5’ GTG TTG ACC CGC TTC CGG CAC TTC CGG AGG ACG AGT 3’
Probes were labelled with $^{35}$S-dATP at the 3' end using terminal deoxynucleotidyl transferase (TDT) enzyme (Roche Diagnostics, UK). Radiolabelled probe and unbound $^{35}$S were separated using Sephadex columns. Tissue sections (one slide from each animal, 3 sections per slide) were incubated with approximately 200 µl radiolabelled probe (70,000-100,000 cpm/µl) in hybridisation buffer. The hybridisation buffer comprised: 50% formamide; 4x SSC; 25 mM phosphate buffer; 10 mM sodium pyrophosphate; 5x Denhardt's solution; 200 µg/ml denatured Salmon sperm DNA; 100 µg/ml polyadenosine 5mg/ml; 120µg/ml heparin; 10% dextran sulphate; 50 mM dithiothreitol. Following overnight incubation, slides were washed (1x SCC room temp. 20 minutes; 1x SSC 55°C 20 minutes, twice; 1x SSC room temp. 1 hour) and allowed to air dry. Slides were then exposed to MR Biomax film (Amersham Biosciences, UK). A slide containing a $^{14}$C microscale standard was included for the purpose of calibration. Films were developed 2-4 weeks later using an automatic developer (Agfa Curix Daylight Processor) and analysed.

**Densitometry**

The TH mRNA signal was used to ensure alignment of the sections between animals such that measurements could be made at a consistent rostrocaudal level of the VTA. Optical densitometry was used to measure mRNA expression. Mean optical density across the VTA measured on left and right sides of a single section was converted to nCi/g of brain tissue using a calibration curve based on the $^{14}$C standard microscale (Amersham Biosciences, UK). Values were then averaged between the left and right sides of the brain.

**Data analysis and statistics**

Repeated blood sampling data are presented as averages over 3 hour blocks starting and finishing at 11:00 h. Data were analysed by two way repeated measures ANOVA with post hoc t test.

For statistical analysis microdialysis data were reduced to four measures. Basal levels refers to the average of the 3 samples before the first K$^+$ stimulation; K1 refers to the peak dopamine concentration measured in the dialysate collected during the first 20 min perfusion with high K$^+$ aCSF; sulpiride response refers to the average of 3 samples taken during the administration of sulpiride (10µM); K2 refers to the peak dopamine concentration measured in the dialysate collected during the second K$^+$ stimulation (i.e. in the presence of sulpiride (10µM)).
Student’s paired t-test was used to determine the significance of treatment effects on basal levels and K1. The impact of treatment on the sulpiride response was analysed by 2-way ANOVA with repeated measures comparing sulpiride response with basal levels in the two treatment groups. The effect of treatments on K2 was analysed using 2-way ANOVA with repeated measures comparing K2 with K1 in the two treatment groups.

For the in situ hybridisation histochemistry data, comparison of mRNA expression between treatment groups was performed using Student’s unpaired t-test.

Data are presented as mean ± sem (n). Significance at the 95% level is quoted.

RESULTS

Corticosterone treatment

Animals appeared healthy throughout the treatment. Weight gain and final weight did not differ between treatment groups. Corticosterone treated animals drank around 32 ml water/day giving them an approximate dose of 5.8 mg/kg/day. Water consumption per cage did not differ significantly between treatment groups.

Blood corticosterone concentrations in the vehicle treated animals showed a clear diurnal rhythm, being highest around the time of lights off (19:00h) and lowest around the time of lights on (see Figure 1). In animals treated with corticosterone in their drinking water there was also a diurnal rhythm but it was flattened compared to the vehicle treated animals with the peak being slightly reduced and the nadir markedly raised. Statistical analysis of these data revealed a significant main effect of time ($F_{7,84}=12.1; p<0.0001$) and no significant main effect of treatment but a significant time x treatment interaction ($F_{7,84}=2.5; p<0.05$). Post hoc t tests showed that corticosterone levels in the treated animals were significantly lower in the sample before the diurnal peak (14:00-17:00 h), and significantly higher in the two samples at the nadir of the rhythm (05:00-08:00 h and 08:00-11:00h).

In the microdialysis and in situ hybridisation histochemistry studies the corticosterone treatment was monitored by measuring adrenal gland weight as this has previously been shown to be reduced by this corticosterone treatment protocol (Fairchild et al, 2003). Corticosterone treatment significantly reduced
adrenal gland weight in both experimental groups. Thus, in the microdialysis study, the adrenal:body weight ratio was $12.2 \times 10^{-5} \pm 0.40 \times 10^{-5}$ (n=12) and $6.8 \times 10^{-5} \pm 0.37 \times 10^{-5}$ (n=12) in the vehicle and corticosterone treated groups, respectively (p<0.0001 unpaired t-test). In the *in situ* hybridisation histochemistry study, the adrenal:body weight ratio was $10.5 \times 10^{-5} \pm 0.31 \times 10^{-5}$ (n=4) and $5.2 \times 10^{-5} \pm 0.84 \times 10^{-5}$ (n=4) in the vehicle and corticosterone treated groups, respectively (p<0.005 unpaired t-test).

**Microdialysis studies: effect of corticosterone treatment on basal and depolarisation-evoked dopamine release**

Basal dopamine levels measured during perfusion of *standard* aCSF were markedly and significantly elevated in corticosterone treated animals in comparison to vehicle treated controls (p<0.01) (see Figures 2 & 3A). Perfusion of *high K*+ aCSF evoked an increase in dialysate dopamine in both treatment groups (Figure 2). However, K1 was significantly greater in the corticosterone treated group than the vehicle treated group (p<0.01) (Figure 3A).

**Microdialysis studies: effect of corticosterone treatment on D₂ receptor-mediated autoinhibition of dopamine release**

The local perfusion of sulpiride, a D₂/₃ receptor antagonist, caused a small decrease in dopamine levels (see Figure 2) which did not reach significance in either treatment group. Thus, two-way ANOVA with repeated measures showed a significant main effect of corticosterone treatment ($F_{[1,13]}=9.5$, p<0.01), but no significant main effect of sulpiride ($F_{[1,13]}=3.1$, n.s), and no significant treatment x sulpiride interaction ($F_{[1,13]}=0.2$, n.s) (Figure 3B). Sulpiride also failed to influence the depolarisation-evoked dopamine release in either treatment group, such that the effect of corticosterone treatment on evoked dopamine release was maintained in the presence of sulpiride (Figure 2). Two-way repeated measures ANOVA, comparing K1 and K2 in the two treatment groups, revealed a significant effect of corticosterone ($F_{[1,12]}=7.9$, p<0.02), but no significant effect of sulpiride ($F_{[1,12]}=1.3$, n.s) and no significant interaction between the two factors ($F_{[1,12]}=0.7$, n.s) (Figure 3C).
Effect of corticosterone treatment on the expression of mRNAs coding for proteins regulating presynaptic dopamine function

The mRNAs coding for TH, VMAT2, DAT, D2 and MAO\textsubscript{A} were densely expressed in the VTA and adjacent dopaminergic cells groups such as the substantia nigra (Figure 4). In our coronal midbrain sections, TH, DAT and D\textsubscript{2} mRNA expression was not seen outside of these regions; VMAT2 and MAO\textsubscript{A} were lightly expressed in the hippocampus and cortex.

In the VTA, the expression of mRNAs coding for both TH and VMAT2 was significantly higher in corticosterone treated animals than in controls (p<0.05) (Figure 4). The expression of D\textsubscript{2} receptor mRNA was also significantly increased in corticosterone treated animals, although the magnitude of the effect was small. In contrast, corticosterone treatment had no significant effect on the expression of mRNAs coding for DAT or MAO\textsubscript{A} in the VTA (Figure 5).

DISCUSSION

In the present study we used a paradigm of corticosterone treatment in rats, which mimicked the flattened and elevated glucocorticoid rhythm seen in patients with affective disorders including bipolar disorder. We have previously shown that this corticosterone treatment protocol, in which animals are allowed free access to corticosterone in their drinking water, elevates plasma corticosterone levels measured at a single time point close to the anticipated nadir and results in an approximate halving of adrenal weight (Fairchild \textit{et al}, 2003). This effect on adrenal weight was confirmed in the animals used for microdialysis and \textit{in situ} hybridisation histochemistry. Furthermore we used repeated blood sampling over 24 hours and confirmed that the treatment protocol flattens the diurnal rhythm (i.e. reduces the amplitude of the rhythm), principally by raising corticosterone levels at the nadir. We observed that, relative to controls, blood concentrations of corticosterone were increased at the end of the dark phase and beginning of the light phase and slightly reduced at the end of the light phase. This profile can be explained by the combination of exogenous and endogenous corticosterone. Thus the intake of exogenous corticosterone which occurs mainly during the dark phase when rats drink (Stephan and Zucker, 1972) has the effect of elevating (exogenous) corticosterone levels during this time period and extending into the early part of the light phase. However, the elevated levels in the light phase also tend to suppress the anticipated rise of
endogenous corticosterone secretion at the end of the light phase so resulting in reduced corticosterone levels at this time. It is worth noting that because of poor aqueous solubility the corticosterone was dissolved in ethanol. Whilst ethanol has been shown to alter aspects of dopamine function, albeit at much higher doses than used here (Ortiz et al., 1995b), this potential confound was controlled for by adding ethanol to the drinking water of the control group.

Flattening of the glucocorticoid rhythm led to marked increases in both the basal dopamine release (i.e. the level measured in the presence of bupropion) and depolarisation-evoked release. We hypothesised that the observed changes in dopaminergic function were the result of transcriptional modulation of key dopaminergic regulatory components within the VTA. Accordingly, our in situ hybridisation studies revealed increases in the expression of mRNAs coding for TH, VMAT2 and D2 in the VTA. While we must acknowledge that attributing functional alterations to changes at the transcriptional level is not straightforward, we can speculate on the most likely mechanism for the functional changes observed.

One possibility is that the corticosterone treated animals had a lower rate of dopamine clearance. However, DAT (the main mechanism of clearance) was continuously blocked by perfusion of the dopamine uptake blocker bupropion, at a concentration shown to maximally inhibit uptake in the nucleus accumbens (L. Ferrie, doctoral thesis, Newcastle University). This means that we can discount the possibility of changes in DAT activity underlying the difference in dopamine dialysate levels. Further support is provided by our in situ data, which showed no effect of corticosterone treatment on DAT mRNA transcription. The use of bupropion throughout our experiment also means that we can regard changes in dialysate dopamine levels as indicative of changes in dopamine release.

A second possibility is that an increase in the basal firing rate of mesocortical dopamine neurones led to an increase in dopamine release. However, the fact that corticosterone treatment resulted in an elevation of depolarisation-evoked (as well as basal) dopamine release, suggests that this is not the case, as depolarisation-evoked neurotransmitter release is not firing rate dependent.
A third potential mechanism involves a decrease in terminal autoreceptor-mediated inhibition of dopamine release. However, we found that the $D_{2/3}$ receptor antagonist sulpiride failed to equalise basal or depolarisation-evoked dopamine release between the two treatment groups. Indeed, in the control group we found a (non-significant) decline in dopamine levels during sulpiride perfusion rather than the expected increase. These data indicate that there is little or no tonic activation of terminal $D_{2/3}$ autoreceptors in the PFC during basal or depolarisation-evoked release. Several previous studies support our data in this respect (Ago et al., 2005; Devoto et al., 2001; Gessa et al., 2000; Moghaddam and Bunney, 1990); but see (Bean and Roth, 1991). Our mRNA data also argue against a decrease in autoreceptor-mediated inhibition as we found an increase in $D_2$ receptor gene transcription in the VTA of corticosterone-treated animals.

The most credible theory to explain our microdialysis findings involves a corticosterone-induced increase in the vesicular dopamine content. Our in situ data show that corticosterone administration increased gene transcription of both the dopamine synthetic enzyme, TH, and the vesicular monoamine transporter, VMAT2. These data are supported by a number of studies showing corticosterone regulation of TH mRNA in the brain (Czyrak et al., 2003), the adrenal medulla and pheochromocytoma cell lines (Baetge et al., 1981; Kumai et al., 2000; Tank et al., 1986). Increases in TH protein expression have also been demonstrated in the VTA following corticosterone administration. Interestingly, other groups have found that rat strains which differ in with respect to their HPA axis activity and reactivity also differ with respect to basal levels of TH in the VTA (Brodkin et al., 1999; Serova et al., 1998), as well as in the responsiveness of TH to administration of corticosterone (Haile et al., 2001; Ortiz et al., 1995a). The presence of a glucocorticoid response element (GRE) on the TH gene (Hagerty et al., 2001) suggests that glucocorticoids modulate TH mRNA transcription directly. Although the effect of glucocorticoids on VMAT2 mRNA expression has not previously been examined, chronic stress has been shown to increase VMAT2 mRNA in medullary noradrenergic cell groups (Rusnak et al., 2001) but to reduce VMAT2 binding in the nucleus accumbens and striatum (Zucker et al., 2005). It is possible that this latter finding represents a functional difference between mesolimbic and mesocortical dopamine systems.
Our hypothesis is that an increase in TH protein, and therefore dopamine synthesis, augments cytosolic dopamine concentrations, increasing the concentration gradient across the vesicular membrane and favouring vesicular accumulation of dopamine. In parallel, an increase in VMAT2 protein promotes active vesicular uptake of dopamine, further increasing vesicular dopamine content. Previous studies have shown that increasing VMAT2 expression increases the amount of dopamine released per depolarisation event; this effect was attributed to an increase in vesicular dopamine content and not an increase in the number of vesicles undergoing exocytosis (Pothos et al. 2000). Our data are consistent with such a mechanism, as depolarisation-evoked stimulation produced the same percentage increase over basal dopamine levels in the corticosterone treated and control groups, suggesting that exocytotic release mechanisms were unaltered. The evidence therefore suggests that increased vesicular content is a likely candidate to explain the increase in dopamine release seen in animals with a flattened glucocorticoid rhythm.

As discussed in the Introduction, mood disorders are associated with a flattening of the diurnal glucocorticoid rhythm and an increase in circulating glucocorticoid (cortisol) levels during the daily nadir (Deuschle et al., 1997; Wong et al., 2000). This glucocorticoid dysrhythmia has also been observed in bipolar disorder patients (Cervantes et al., 2001; Linkowski, 2003). Our findings of marked alterations in aspects of dopaminergic neurotransmission suggest that the same alterations may occur in patients with bipolar disorder, and other affective disorders associated with a flattened glucocorticoid rhythm. In this respect it is of note that clinical post-mortem brain studies have shown increased TH protein expression in the locus coeruleus of patients with major depression (Zhu et al., 1999) and increased VMAT2 binding in both the thalamus and ventral midbrain of bipolar disorder patients (Zubieta et al., 2001).

The finding of increased dopamine release in the PFC suggests a causal link between the neuroendocrine abnormalities observed in mood disorders, and some of the cognitive symptoms of these conditions: the PFC plays a key role in cognitive functions including working memory, selective attention, goal directed behaviour, and behavioural inhibition (Dalley et al., 2008; Nagahama et al., 1996; Wager and Smith, 2003) with PFC dopaminergic neurotransmission strongly influencing these functions (Arnsten and Li, 2005;
Dalley et al, 2008; Sawaguchi and Goldman-Rakic, 1994). In this context, our data can be seen to offer further support to the targeting of dopaminergic and glucocorticoid signalling pathways for the treatment of affective disorders.

DISCLOSURE/CONFLICTS OF INTEREST
The authors have no conflicts of interest.
SEG, AHY and CDI have been the recipients of unrestricted research grant funding from Organon Laboratories and are co-inventors on a patent application relating to the therapeutic use of glucocorticoid receptor antagonists.

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Figure 1. Effect of corticosterone administration on the 24 h blood corticosterone profile. Blood corticosterone levels averaged in 3 h blocks in rats treated for 14 d with corticosterone (50 µg/ml) or vehicle (0.5% ethanol) in the drinking water. Data are mean ± s.e.m (n=6 (vehicle) and 8 (corticosterone). Bar represents the period of darkness. * p<0.05 unpaired t test following significant two way ANOVA. See text for full statistical analysis.
Figure 2. Effect of corticosterone treatment on basal dialysate dopamine. Time course of changes in dialysate levels of DA in the vehicle treated (open circles) and corticosterone treated (closed circles) groups. Data are mean ± s.e.m. Only animals for which the whole time course data set was available are included in this figure (n=6 and 7 per group). Bars indicate the application of aCSF solutions of different compositions via the dialysis probe. Open bar indicates aCSF containing bupropion (30 µM), hatched bar indicates aCSF containing sulpiride (10 µM), filled bars indicate aCSF with 100 mM K⁺. See Figures 2 and 3 and Results section for statistical analysis.
Figure 3. Effect of corticosterone treatment on dopamine in dialysates of the prefrontal cortex.

Part A) shows the effect of corticosterone treatment on basal and depolarisation-evoked dopamine. ‘Basal’ is the average of the three baseline samples and ‘K1’ is the dopamine level in the dialysate collected during the perfusion of 100mM K⁺. *p<0.01 paired t test corticosterone vs vehicle. Part B) shows the effect of corticosterone treatment on the response to sulpiride. ‘Before’ is the average of three samples before perfusion of sulpiride and ‘sulpiride’ is to an average of the three samples collected during perfusion of sulpiride. Part C) shows the effect of corticosterone on depolarisation-evoked DA levels in the absence and presence of sulpiride. K1’ is the peak level during the perfusion of K⁺ 100mM in the absence of sulpiride and ‘K2’ is the peak DA level during the perfusion of K⁺ 100mM in the presence of sulpiride (10 µM). Data from the vehicle group is shown in the open bars, the corticosterone treated group is shown in the hatched bars. Data are mean ± sem. A) n=11 and 12 per group B) n=8 and 9 per group, C) n=7 and 7 per group. See Results text for full statistical analysis.
Figure 4. Expression of selected mRNA species in the VTA. Sample autoradiograms from coronal sections of midbrain (from vehicle treated animals) showing the typical observed distribution of expression of mRNAs encoding (a) tyrosine hydroxylase (TH), (b) VMAT2, (c) D₂ receptor, (d) dopamine transporter (DAT), and (e) monoamine oxidase A (MAOₐ); (f) shows a plate from the Rat Brain Atlas (Paxinos and Watson, 1986) at the corresponding level with the VTA shaded in grey.
Figure 5. **Expression of mRNA species in the VTA.** Expression of mRNAs encoding tyrosine hydroxylase (TH), VMAT2, the D₂ receptor, dopamine transporter (DAT), and monoamine oxidase A (MAO_A) in the VTA in vehicle treated (open bars) and corticosterone treated (hatched bars) groups. Data are mean ± sem (n=8/group). * p<0.01 unpaired t test.